Journal of Chromatography, 162 (1979) 351—366
Biomedical Applications

CHROMBIO. 273

RELIABLE ROUTINE METHOD FOR THE DETERMINATION OF PLASMA AMITRIPTYLINE AND NORTRIPTYLINE BY GAS CHROMATOGRAPHY

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(First received April 17th, 1978; revised manuscript received September 25th, 1978)

SUMMARY

A gas chromatographic method has been developed for the determination of amitriptyline and nortriptyline in plasma. OV-17 is used in a 1 m long packed column, with a flame ionization detector and an electronic integrator. Five internal standards are added. The base-specific extraction procedure and the method of calibrating the chromatograph are described in detail. The accuracy, precision and reliability of the method are demonstrated by the results of nearly 700 determinations of each drug, at concentrations ranging from 5 to 400 ng/ml in the plasma. An interlaboratory comparison with a double radioactive isotope derivative assay for nortriptyline has also shown satisfactory agreement.

INTRODUCTION

The routine determination of amitriptyline and its demethylated metabolite nortriptyline in plasma remains difficult although numerous methods have been described in the last two years. Plasma levels in patients treated with amitriptyline may reach only 20 ng/ml, and in pharmacokinetic studies using single doses, concentrations of a few ng/ml need to be measured. The tendency of tricyclic antidepressants to adsorb onto surfaces complicates extraction procedures at these low concentrations, and contamination with interfering materials occurs easily. Routine reliability is particularly difficult to achieve.

Gas chromatography (GC) with mass spectrometry (MS) [1,2] seems to offer the best available solution of the main problems. Garland [2] used deuterated amitriptyline and nortriptyline as internal standards and showed, as expected, that the ratios of labelled to unlabelled compounds were unchanged by extraction and chromatographic procedures. Thus the deuterated compounds acted as ideal internal standards and controlled for losses in chromatography as well as in extraction. The chemical ionization mass spectrometer was highly

sensitive and provided considerable (though not absolute) specificity of detection. Coefficients of variation were under 4% for samples containing only about 6 ng of nortriptyline and 9 ng of amitriptyline. Garland found that absolute recoveries of both drugs varied, and at the lower concentrations the percentage recoveries were smaller and more variable. He attributed these erratic losses in part to adsorption and stressed the consequent importance of using isotopic internal standards.

Unfortunately many investigators find the cost of MS equipment prohibitive.

Internal standards labelled with ^{14}C have been used for double radioactive isotope methods, in which tricyclic and related antidepressants are determined by acetylation with tritiated acetic anhydride. However, only the secondary amines such as maprotiline [3], nortriptyline [4] and desmethylclomipramine [5] can be directly acetylated. Clomipramine has been acetylated after chemical demethylation [5], but the procedure is laborious and sensitivity is lost. Furthermore, the radioactive acetylation methods lack specificity: at best, thin-layer chromatography is used to separate the desired compound from other substances that will acetylate, such as natural components of plasma, other drugs and drug metabolites. Carnis et al. [5] found that even after a base-specific extraction followed by thin-layer chromatography, the acetylation of plasma components limited the accuracy of the method.

Radioimmunoassay [6,7] has recently been introduced for amitriptyline and nortriptyline. The available antisera do not distinguish between these two drugs, so that both are estimated together [6], or alternatively they may be separated [7] before assay by a differential extraction comparable with the procedure needed for chromatographic methods.

All other published methods for determining amitriptyline with acceptable sensitivity and specificity have been chromatographic, using internal standards chemically different from the drugs estimated. Most authors have used GC with electron capture detectors [8—10], flame ionization detectors [11—14], or nitrogen detectors [15—23]. Some recent methods [24—26] employ high-performance liquid chromatography, but so far this technique does not seem to offer appreciable advantage: the sensitivity is no greater and the problems of quantitative extraction are unchanged. These limitations also apply to high-performance thin-layer chromatography [27], an interesting new technique in which the time taken for chromatography is much reduced.

This paper describes a method for estimating amitriptyline (AT) and nortriptyline (NT) in plasma by GC. The method is conventional, using only minimum equipment including flame ionization detectors. The detailed procedure has been very fully investigated and developed to provide a high degree of reliability. The present version has been in routine use for ten months and the results of nearly 700 determinations of each drug are reported here.

The compounds are extracted from alkaline plasma into heptane and thence into HCl, which is made alkaline and re-extracted with 50 μl heptane. A 10-μl aliquot of this extract is injected into the chromatograph without derivative formation. We follow Cooper et al. [20] in avoiding the evaporation of any solvent, and Jorgensen [15] in adding a low-molecular-weight amine (in our case diethylamine) which prevents adsorption of the tricyclic bases to glass.

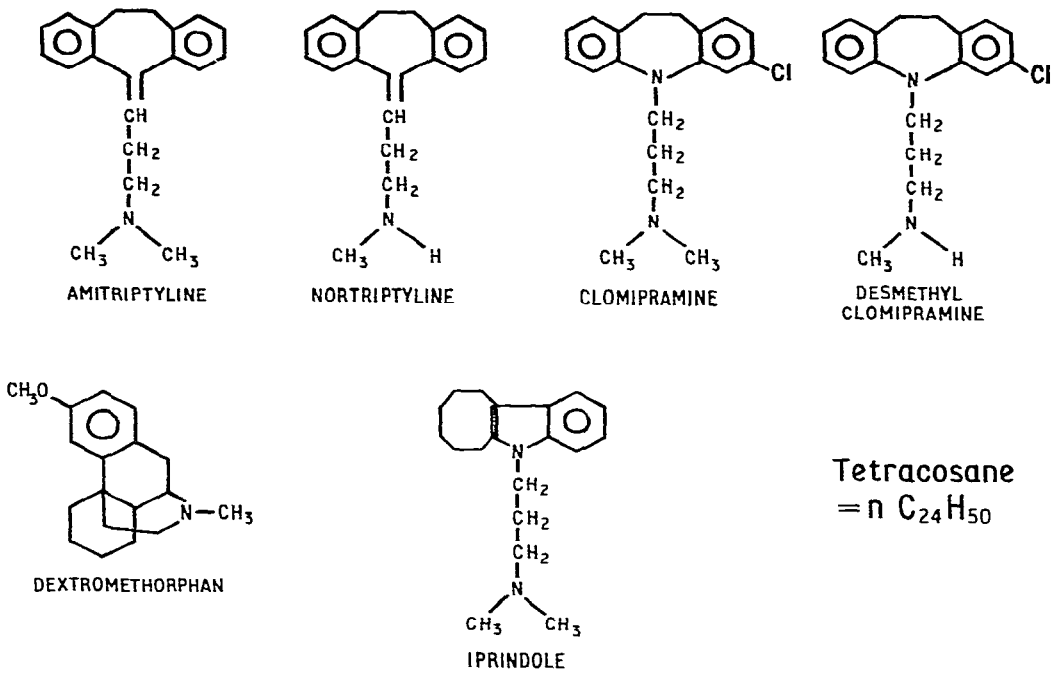


Fig. 1. Molecular structures of AT, NT, internal standards and tetracosane.

Four internal standards are used simultaneously: dextromethorphan and clomipramine are added as hydrochlorides in aqueous solution to the plasma sample; iprindole and desmethylclomipramine are added as bases in heptane, to the first extraction step. A fifth compound, tetracosane, is included in the 50 μl of solvent used in the final extraction, to allow calculation of the over-all yield in the processing of each sample. Fig. 1 shows the molecular structures of these compounds, AT, NT and tetracosane. Dextromethorphan is normally chosen as the primary internal standard for calculating AT and NT concentrations, but the additional internal standards have been found helpful both in developing the method and in routine work.

The chromatogram usually shows all four basic standards in approximately the proportions in which they were added. However, on some occasions the proportions differ, giving warning that the estimation must be carefully considered. Patients' plasma often contains other basic drugs, prescribed or otherwise. These may interfere on the chromatogram with internal standards, and it is always possible that the patient has taken a drug that is used as an internal standard. Furthermore, in routine work peaks from accidental contamination at the ng level are not unknown. In all these instances the presence of several internal standards in fixed ratios usually allows the estimation to be rescued; the procedure insures against errors that might otherwise pass undetected.

MATERIALS AND METHODS

Reagents

n-Heptane (99.5%, international pesticide specification), *n*-pentane (99%)

and diethylamine (analytical reagent grade) were obtained from BDH (Poole, Great Britain) and all were distilled before use.

Compounds

Tetracosane was obtained from Aldrich (Milwaukee, Wisc., U.S.A.). Drug compounds were kindly donated by the following firms: amitriptyline hydrochloride by Merck, Sharpe & Dohme (Hoddesdon, Great Britain), nortriptyline hydrochloride by E.R. Squibb and Sons (Twickenham, Great Britain), dextromethorphan hydrobromide by Parke Davis (Pontypool, Great Britain), iprindole hydrochloride by Wyeth Labs. (Maidenhead, Great Britain), clomipramine and desmethyl clomipramine hydrochlorides by Geigy Pharmaceuticals (Macclesfield, Great Britain).

Preparation of standard solutions

Aqueous salt solutions. An amount of each drug equivalent to 50 mg base is weighed, and dissolved in 0.01 *M* HCl to give 200 ml stock solution. Dilutions are prepared as follows:

(i) Internal standard mixture, containing dextromethorphan (1 μg/ml) and clomipramine (2 μg/ml) in 0.01 *M* HCl.

(ii) Amitriptyline (1 μg/ml) and nortriptyline (1 μg/ml) in 0.01 *M* HCl. This solution is added to drug-free plasma to give standard samples.

The aqueous solutions are stored at 4° and all except iprindole remain stable for several months. Iprindole is best kept as a base (see below).

Base solutions in heptane. A standard solution of the basic form of each drug is prepared from 25 ml of the stock salt solution, by extraction with 25 ml heptane and 500 μl diethylamine in a glass-stoppered tube for 45 min on a rotary tumbler. Each solution contains 0.25 mg base per ml, in heptane containing about 0.5% of diethylamine.

Tetracosane is weighed, and dissolved in heptane containing 0.5% (v/v) of diethylamine. The stock solution contains 100 μg/ml and is stable at room temperature for many months. A working solution containing 5 μg/ml is prepared by dilution with the same solvent.

Mixtures of bases are prepared in this solvent, for calibrating the chromatograph. Ten microlitres of each contain 50 ng tetracosane, 75 ng dextromethorphan, 100 ng iprindole and amounts of AT, NT, clomipramine and desmethylclomipramine ranging from 1 to 150 ng.

An internal standard base mixture is prepared, containing iprindole (0.75 μg/ml) and desmethylclomipramine (1 μg/ml) in the same solvent.

All the base solutions are stable at room temperature for several months.

Glassware

The extraction of each plasma sample requires three glass-stoppered tubes: a 35-ml centrifuge tube, a 15-ml test-tube and a 10-ml conical centrifuge tube. After use, each tube is rinsed with ethanol and then distilled water. The conical tubes are then soaked in chromic acid overnight, rinsed with distilled water, soaked in 2 *M* NaOH for at least 1 h and rinsed again in distilled water. All tubes are finally soaked overnight in Decon 90 detergent (Decon Labs., Brighton, Great Britain), rinsed with distilled water and dried in an oven.

The 15-ml tubes and conical tubes, with their stoppers, are silanised

by standing for 30 min in 2.5% dimethyldichlorosilane in toluene, rinsed in toluene, soaked in methanol for 10 min, rinsed twice with methanol and dried. These tubes are rinsed with pentane immediately before use.

Extraction of plasma

Into a 35-ml tube is pipetted 1.0 ml of a solution prepared by diluting 50 ml 4 *M* NaOH and 20 ml diethylamine to 200 ml with water. The tube is stoppered and rotated in the hand until the whole surface has been wetted with alkali.

To the plasma sample (1—10 ml) in a polystyrene tube are added 500 μl of the aqueous internal standard solution of dextromethorphan and clomipramine. The sample is mixed and then washed quantitatively into the 35 ml tube with water. One millilitre of the internal standard base mixture containing iprindole and desmethylclomipramine is added, and 10 ml heptane. Water is then run in until the tube is almost full. It is stoppered and mixed at 30 rpm on a rotary tumbler for 30 min. The dilution of the plasma and the gentle tumbling action prevent emulsification.

After centrifuging (without stopper) for 15 min at 2000 *g*, the heptane layer is transferred to a 15 ml tube, using a Pasteur pipette that has been rinsed first with diethylamine and then with heptane. (Between successive samples the pipette is rinsed with heptane only.) A 2-ml volume of 0.1 *M* HCl is added, the tube tumbled at 30 rpm for 10 min and then centrifuged briefly with the stopper in place. Most of the heptane is removed by aspiration.

Into a conical tube are pipetted 300 μl of a solution prepared by diluting 50 ml of 4 *M* NaOH and 30 ml diethylamine to 200 ml with water. The whole surface is wetted with this solution as before. The acid layer from the 15-ml tube is transferred to this conical tube, using a Pasteur pipette rinsed in 0.1 *M* HCl before use and between samples. Care is taken to avoid transferring any heptane layer.

A 50-μl volume of the working tetracosane solution is then added and the tube vibrated on a Whirlimixer for 10 sec. After centrifuging briefly with the stopper in place, most of the lower layer is removed with a Pasteur pipette previously rinsed in diethylamine and then in pentane. Between one sample and the next the pipette is simply wiped carefully on paper tissue. The tube is centrifuged again and all the remaining aqueous layer removed, using a drawn-out capillary pipette rinsed in the same way as the previous one. Ten microlitres of the resulting extract are injected into the chromatograph. This is usually done the same day, but the extracts can be kept at room temperature until the following day, without change.

Gas chromatography

The Pye-Unicam GCV chromatograph is fitted with dual columns, flame ionization detectors and amplifiers, and a Philips PM8221 two-pen chart recorder. Pye-Unicam DP88 integrators are used to measure retention times and peak areas.

Columns are silanised glass, 1 m × 4 mm I.D., packed with 10% OV-17 on Gas-Chrom Q 80—100 mesh. It is important to leave space above the packing at the inlet end, so that the injection needle does not enter the packing material.

(If the needle regularly penetrates into the packing, the resolution of the column deteriorates rapidly.) The packing is supplied by Jones Chromatography (Llanbradach, Great Britain). Columns are conditioned at 270° for about 45 h with low carrier flow.

The carrier gas is nitrogen, flow-rate 75 ml/min. Hydrogen and air flow-rates are 50 ml/min and 300 ml/min, respectively. Working temperatures are: column oven 230°, injection oven 230°, detector oven 270°.

All injections are of 10 μl volume, from syringes manufactured by Scientific Glass Engineering (Melbourne, Australia).

RESULTS AND DISCUSSION

Gas chromatography

The standard mixed base solutions are used daily to check the chromatographic system. Injection of large amounts (one hundred to several hundred ng) of the various compounds gives peak area ratios proportional to the relative amounts present. However, when smaller amounts of AT and NT are injected their peak areas are lower than would be expected from simple proportion. Each day, the base solution containing 150 ng of AT and NT per 10 μl is first injected several times. Peak area ratios are calculated, and seldom differ by more than 2% from the mean values shown in Table I. Mixtures containing smaller amounts of AT and NT are then injected and, each time, the peak areas of the internal standards are used to calculate the expected areas of AT and NT by simple proportion. The actual areas are subtracted from expected areas to find the losses of AT and NT in chromatography. These losses are small but significant; they differ from day to day and between columns, but are usually between 0.5 and 1 ng of AT and between 1 and 2 ng of NT. When mixtures containing different amounts (1—30 ng) of AT and NT were compared, it was found that the loss of each compound in chromatography was approximately independent of the quantity injected. Routinely, 7.5 ng of AT and NT are injected several times for the determination of area losses.

Plasma extracts are then injected and the area of each AT and NT peak is corrected by adding the loss already estimated. Corrected areas, together with

TABLE I

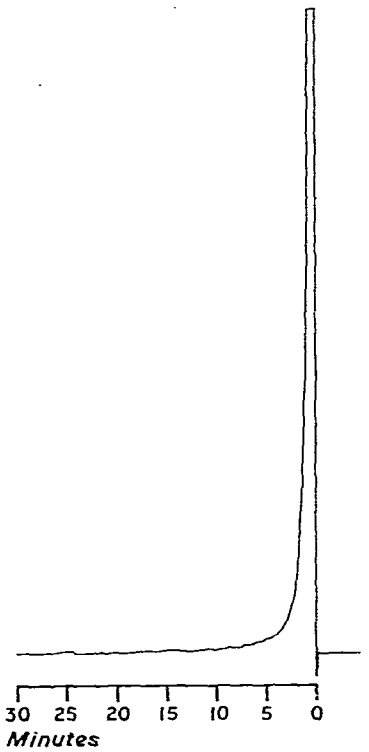
RELATIVE RETENTION TIMES AND PEAK AREAS

Compound	Relative retention time (AT = 1)	Peak area ratio for equal weights of compounds (AT = 1)
Tetracosane	0.68	1.05
Dextromethorphan	0.84(5)	0.87
Amitriptyline	1.00	1.00
Nortriptyline	1.16(5)	0.96
Iprindole	1.53	0.87
Clomipramine	2.00	0.82
Desmethylclomipramine	2.40	0.76

the area ratios determined at 150 ng, are used to calculate AT and NT concentrations in the plasma sample by simple proportion. It is thus assumed that the relative amounts of the various compounds in the extract are the same as those in the original plasma sample after addition of internal standards.

The ratios of the peak areas of the different internal standards in each extract are routinely calculated, and the ratio of dextromethorphan to tetracosane is used to estimate the over-all yield of the extraction process, which normally averages between 70 and 75%. Thus 10 μl of plasma extract contains the amounts of AT and NT in approximately one-seventh of the plasma sample, together with about 50 ng tetracosane, 75 ng dextromethorphan, 110 ng iprindole, 150 ng clomipramine and 150 ng desmethylclomipramine. A loss of 1 ng AT or NT in chromatography corresponds to about 1.5 ng/ml in a 5-ml plasma sample.

Fig. 2 shows a chromatogram from 5 ml of blank plasma carried through the usual procedure, but without the addition of internal standards or tetracosane. Fig. 3 shows a chromatogram from 4.8 ml of the plasma of a subject who had taken a single dose of 100 mg amitriptyline 4 h previously. The calculated drug concentrations were AT 71 ng/ml and NT 13 ng/ml. The retention time of AT



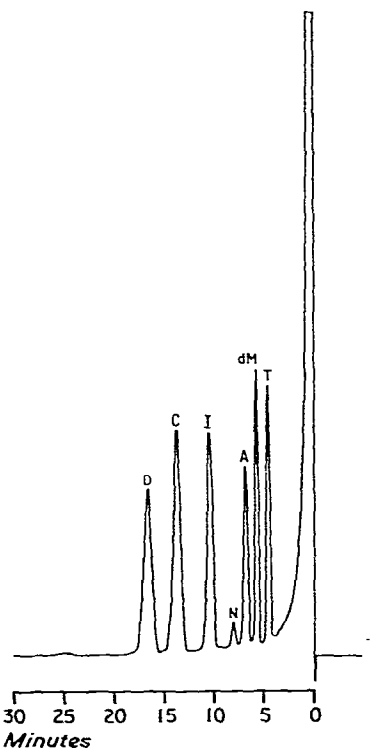


Fig. 2. Chromatogram from 5 ml blank plasma carried through the extraction procedure. Attenuation the same as for Fig. 3.

Fig. 3. Chromatogram from 4.8 ml of plasma from a subject who had taken a single dose of 100 mg amitriptyline 4 h previously. Calculated drug concentrations were AT 71 ng/ml, and NT 13 ng/ml. Peaks: T, tetracosane; dM, dextromethorphan; A, amitriptyline; N, nortriptyline; I, iprindole; C, clomipramine; D, desmethylclomipramine.

was 6.9 min; it differs between columns but is usually between 6 and 7 min. The retention times of the other compounds relative to AT are shown in Table I.

A column packing material containing a high proportion (10%) of liquid phase has been adopted, because all packings with lower percentages that were tried gave greater losses of AT and especially of NT. Some batches of the current packing obtained from various other suppliers were also found to be inadequate, but with good batches sufficient resolution is obtained in 1-m columns, which give about 2000 theoretical plates with these compounds.

Assay results

Each batch of 12 samples processed usually contains two of drug-free plasma. One is a blank, and known amounts of AT and NT are added to the other. Table II shows the results of 120 such determinations, most of which were carried out on different days. Usually, equal amounts of AT and NT were present.

The two drugs showed no interaction in the estimation. In 5 ml of plasma, 20 ng/ml of added AT gave the same result when 20 ng/ml of NT were also added as when no NT was present. Similarly, the results with 20 ng NT per ml were unaffected by the presence or absence of 200 ng AT per ml.

Neither the amount of plasma present nor the anticoagulant used affected the assay. Ten millilitres of plasma, either citrated from the blood bank or heparin-treated from volunteers, 1 ml plasma of either type, and water were compared. Firstly 1000 ng of AT and NT were added to each sample and then

TABLE II

RESULTS OF DETERMINATIONS ON 5-ml PLASMA SAMPLES WITH ADDED DRUGS

Number of estimations = 20 for each concentration.

Drug	Amount added (ng/ml)	Amount found (ng/ml)		Coefficient of variation (%)
		Mean	S.D.	
Amitriptyline				
	5	5.2	0.47	9.1
	10	10.0	0.70	7.0
	20	19.1	0.69	3.6
	40	39.4	1.25	3.2
	100	99.9	2.70	2.7
	400	415	11.2	2.7
Nortriptyline				
	5	5.7	0.60	10.7
	10	10.6	0.96	9.0
	20	19.5	0.87	4.5
	40	39.2	1.08	2.8
	100	98.0	2.65	2.7
	400	414	10.8	2.6

the experiment was repeated with 75 ng per sample. No differences were found at either drug level.

For the determinations summarized in Table II, AT and NT concentrations were calculated as explained above, assuming that the extraction yield of each drug, at all concentrations from 5 to 400 ng/ml, was identical with the yield from 100 ng dextromethorphan per ml in the same sample. The close agreement in Table II between the amounts of drug added and the mean amounts estimated shows that the assumption is justified and that attempts to calibrate the extraction process, by using the results of extracted standard samples to calculate the results for unknown samples, would be unlikely to improve accuracy. This is satisfactory. If extraction yields vary with concentration or differ between different compounds they are likely to be inherently unstable, making any corrections unreliable. Our efforts to improve the extraction procedure in order to eliminate such variations appear to have been successful, within the limits shown in Table II.

Table II shows that when the AT concentration is 40 ng/ml or greater, coefficients of variation are effectively constant at about 3%. At lower levels, coefficients of variation rise: the standard deviation falls to about 0.7 ng/ml (3.5 ng per sample) but thereafter remains constant. Variations in the losses in chromatography are probably responsible for much of this residual standard deviation. NT gives figures very similar to those for AT: standard deviations at low concentrations are marginally higher, as are the losses in chromatography. The minimum amount of either drug that can be detected with certainty is about 15 or 20 ng per sample.

Treatment of blood samples

Blood is collected in heparin-coated polystyrene tubes and usually centrifuged soon afterwards. The plasma is transferred to fresh polystyrene tubes and kept frozen until analysed. However, when blood samples are taken in the evening they are left at room temperature until they are centrifuged next morning, and some samples of plasma are received by post, taking up to seven days to arrive.

Patients' blood samples were used for the following six experiments:

(1) Ten blood samples left at room temperature for 24 h before centrifuging were found to give the same plasma concentrations of AT and NT as duplicate blood samples centrifuged immediately after collection.

(2) Three plasma samples left at room temperature for ten days gave the same AT and NT concentrations as duplicate plasma samples frozen immediately, and analysed with the others ten days later.

(3) Eight plasma samples frozen and left five to seven months in the freezer before analysis showed little or no loss of AT and NT, compared with duplicate samples analysed at the beginning. The nominal mean losses were 6% AT and 4% NT, which were probably within the errors of the estimation over this time interval.

(4) On storage in the freezer, plasma samples develop precipitates. These precipitates were shown not to adsorb appreciable amounts of AT or NT from the plasma. Two samples analysed complete with precipitate gave the same results as duplicate samples of clear supernatant after centrifuging. Analysis of

TABLE III

PRECISION OF AMITRIPTYLINE ESTIMATIONS FROM DUPLICATE ANALYSES OF PLASMA FROM PATIENTS AND NORMAL VOLUNTEERS

Range (ng/ml)	0–10	10–20	20–30	30–50	50–70	70–100	100–150	over 150
Mean	6.3	14.8	25	40	59	83	117	168
Number of pairs	28	27	13	42	60	44	33	5
S.D.	0.54	0.58	1.4	2.1	2.5	4.3	5.5	8.8
Coefficient of variation (%)	8.5	4.0	5.6	5.3	4.2	5.2	4.7	5.2

TABLE IV

PRECISION OF NORTRIPTYLINE ESTIMATIONS FROM DUPLICATE ANALYSES OF PLASMA FROM PATIENTS AND NORMAL VOLUNTEERS

Range (ng/ml)	0–10	10–20	20–30	30–50	50–70	70–100	100–150	150–200	over 200
Mean	6.2	14.3	24	40	59	86	123	168	251
Number of pairs	35	49	15	22	38	41	33	16	18
S.D.	0.74	0.65	1.55	3.1	2.5	4.3	4.8	7.7	10.4
Coefficient of variation (%)	11.9	4.5	6.4	7.6	4.2	5.0	3.9	4.6	4.1

the precipitates from 24 ml of old stored plasma showed only traces of AT and NT.

(5) Adsorption of AT and NT from plasma on to glass pipettes or polystyrene tubes was shown to be negligible: two samples of clear centrifuged plasma were each sucked up and down in five ordinary 2-ml glass pipettes in series. Two further samples were each poured into five polystyrene tubes, from one to the other in series. No loss of AT or NT could be detected. The binding of the drugs to plasma proteins probably prevents adsorption on to other surfaces.

(6) In view of the problems experienced with storing dilute aqueous solutions of AT and NT in polystyrene tubes in the freezer (see later), plasma was tested for the effect of freezing and thawing in these tubes. Plasma was poured into a clean polystyrene tube and frozen. Next day, it was thawed, poured into a fresh tube and refrozen. This process was carried out five times altogether, on each of three samples. There was no change in AT or NT concentrations, compared with duplicate samples frozen once only and kept frozen.

Duplicate determinations on plasma samples

The precision of the method was confirmed by analysis of the results of duplicate determinations, using the method of Snedecor [28]. Plasma samples were either from psychiatric patients being treated with AT or NT, or from normal subjects who had ingested single doses of AT. The volume of plasma used in each estimation ranged from 3 to 6.5 ml, with a mean of about 4 ml. Duplicates were always estimated on different days. The results are given in Tables III and IV. (One pair of duplicates with grossly discrepant values for both drugs has been omitted.)

The coefficients of variation are satisfactory for most purposes but are slightly larger at high concentrations than those of Table II. These duplicate determinations at the higher concentrations (patients in steady state) were carried out over a period of ten months, during which period improvements were being made to the procedures. The estimations in Table II, however, and those in the low-concentration groups of Tables III and IV (single-dose studies), were carried out within the last three months. These improvements were probably responsible for the better precision obtained.

Additional peaks on the chromatogram

Normal plasma contributes no peaks (Fig. 2). In order to avoid contamination during extraction, extreme cleanliness of the glassware, and particularly of the final conical tube, is essential. The present extraction method replaced an earlier technique that included the evaporation of 5 ml pentane to dryness. Not only did recoveries of the compounds become more reproducible, but background peaks were largely eliminated.

Metabolites of AT and NT. Peaks with retention times of 1.89 and 2.20 relative to AT are usually found in small amounts in plasma extracts from patients treated with AT, but never in extracts from drug-free plasma with or without added drugs. Patients treated with NT show only the later peak. These compounds are probably 10-hydroxy-AT and 10-hydroxy-NT, respectively, well-known metabolites of AT and NT [29]. At a column temperature of 212° their relative retentions were found to be 1.98 and 2.41, respectively,

in good agreement with the values of 1.96 and 2.38 reported by Hucker and Stauffer [13] for the authentic compounds on OV-17 at this temperature.

These peaks are not resolved from clomipramine and desmethylclomipramine, respectively. However, if the amounts present are large enough to add significantly to the measured peak areas of these internal standards, the metabolites can be clearly seen as shoulders on the leading edges of the respective standard peaks. In such samples, the value of clomipramine and/or desmethylclomipramine as internal standards is of course reduced.

Contaminants. Two extraneous peaks still often appear on the chromatograms, at retention times of 2.12 and 3.6 relative to AT. These substances probably enter during the last stage of the extraction and all efforts to exclude them completely have failed. The later peak merely increases the necessary time interval between injections, but the earlier one forms a shoulder on the rear edge of the clomipramine peak. As with 10-hydroxy AT, it can be seen if there is enough present to add appreciably to the integrated clomipramine area.

If any heptane layer is accidentally transferred into the conical tube, it contributes a peak from plasma constituents, at a retention time of 10 relative to AT. This peak is likely to intrude upon the chromatograms of subsequent samples.

Other drugs

The relative retention times of 29 basic drugs and some of their metabolites are given in Table V. Retention time depends broadly on molecular weight and polarity. However, small, highly polar amines that might give peaks in the region of interest will be excluded by the extraction process. Usually, metabolites are more polar than the parent drug and hence have longer retention times; the demethylation of tertiary amines and the hydroxylation of hydrocarbon structures both have this effect. N-Oxidation of tertiary amines can shorten retention time [15] but the oxide is not basic and so cannot appear in the plasma extract. It seems possible, however, that where a drug is an ester, metabolites formed after hydrolysis might have shorter retention times than the parent compound.

The information in Table V suggests that large numbers of benzodiazepines and phenothiazines can be eliminated as sources of interference with dextromethorphan, AT or NT. Medazepam, the least polar benzodiazepine that we have encountered, overlaps with iprindole on the chromatogram. The introduction of a carbonyl group, to give diazepam, considerably increases retention time as expected. All the other benzodiazepines familiar to us, including oxazepam, lorazepam, flurazepam and clonazepam, are more polar and/or larger than diazepam and are therefore extremely unlikely to interfere. Similarly, the phenothiazines promethazine, trimeprazine and promazine, appear in the iprindole region of the chromatogram. The introduction of a chlorine atom, giving chlorpromazine, increases retention time as expected. Thioridazine, with an extra ring on the basic side-chain, comes very much later. All the phenothiazines that we have encountered, including fluphenazine, trifluoperazine, perphenazine and levomepromazine, are larger and/or more polar than chlorpromazine.

Haloperidol is also large and polar and was excluded by Bailey and Jatlow

TABLE V

RELATIVE RETENTION TIMES OF TWENTY-NINE BASIC DRUGS

Drug	Relative retention time (AT=1)	Drug	Relative retention time (AT=1)
Lidocaine*	0.36	Promethazine	1.45
Orphenadrine*(1)	0.41	Trimeprazine	1.48
Diphenyl pyraline	0.75	Medazepam	1.48
Bromodiphenhydramine	0.85	Iprindole	1.53
Procyclidine*(2)	0.88	Benztropine	1.55
Dextropropoxyphene*(3)	0.88	Maprotiline	1.71
Propranolol	0.92	Promazine*(6)	1.76
Butriptyline	0.93	Dothiepin	2.06
Trimipramine*(4)	1.04	Antazoline	2.14
Imipramine	1.11	Chlorpromazine*	2.83
Atropine	1.14	Diazepam*	3.16
Mianserin*(5)	1.20	Nitrazepam	11.7
Doxepin	1.22	Chlordiazepoxide	15.3
Desipramine	1.32	Thioridazine*	21.9
Protriptyline	1.35		

*These drugs have been identified in extracts prepared as usual from patients' plasma. Metabolite peaks have also been found, with retention times relative to AT, as follows: (1) 0.44; (2) 1.00 and 1.33; (3) 0.81 and 4.00; (4) 1.27; (5) 1.57; (6) 2.18.

[19], who chromatographed 47 basic drugs on OV-17 at 235°. Of these, only atropine, scopolamine, doxepin and imipramine were reported to interfere with AT or NT peaks, although no retention times were given. The remaining 43 non-interfering drugs include 36 that do not appear in Tables I or V of the present paper.

Four further drugs that have retention times within 10% of those of AT or NT are shown in Table V. All but propranolol are themselves antidepressants and are therefore usually avoidable in combination with AT or NT. No drugs have yet been found whose retention times are so close to those of AT or NT that their presence would pass undetected. However, it is necessary to remain aware of the possibility of interference and to test any suspect basic drug that a patient is known to be taking. Pharmaceutical preparations can readily be treated with 0.1 *M* HCl and the supernatant solution made alkaline and extracted with heptane for injection into the chromatograph.

Interference by metabolites, particularly of drugs with retention times shorter than that of AT, remains more difficult to eliminate. Such metabolites can only be sought in extracts of the plasma of subjects who are taking the drug in question without AT or NT. We have found that a metabolite of procyclidine gives a very small peak coincident with that of AT; furthermore, the peak of procyclidine itself is close to that of AT and often very large.

Comparison with double radioactive isotope assay of nortriptyline

Twenty-four frozen plasma samples whose duplicates had been analysed for NT by the double isotope derivative dilution assay [4] were sent by air from Melbourne, Australia. They were estimated by a slightly earlier version of our GLC method, and the subsequent comparison of results has been reported elsewhere [30]. Agreement was satisfactory: the mean ratio between the two results for each sample was 0.99, and the joint coefficient of variation was 8.6%.

Development of the method

The hydrocarbon tetracosane, with its ideal chromatographic behaviour, has been found useful in overcoming problems encountered in chromatography, as well as for checking the over-all yield of the extraction process.

The four basic internal standards were chosen by trial and error as lipophilic bases with conveniently spaced retention times. Several compounds more closely related to AT and NT were tried and abandoned: protriptyline on account of the very active adsorption of its base on chromatography columns and on glassware; and all the tricyclic drugs in Table V from butriptyline to desipramine inclusive, because of inadequate separation from AT or NT. We used clomipramine as the primary standard until we discovered the presence of hydroxy-AT in many plasma extracts. This peak interferes with clomipramine and is also close to maprotiline. Desmethylclomipramine is more variable in its extraction yield than are the other compounds used, and suffers interference from hydroxy-NT. Iprindole has the slight disadvantage that it is used in heptane solution, being unstable on storage in dilute acid. Dextromethorphan remains the best internal standard that we have found, in spite of the difference in chemical structure between it and the tricyclic drugs.

We find it useful to add the four internal standards in two separate solutions, to guard against pipetting errors, which would cause a characteristic pattern of peak area ratios.

AT and NT bases were found to adsorb very markedly from solution in pure heptane, particularly on to the glass barrel and metal needle of the injection syringe. Much more NT than AT was adsorbed. The addition of 0.5% diethylamine prevented the effect, and in this mixed solvent all six bases used can be diluted, pipetted, etc., without significant losses. In the extraction, the 35-ml tube does not adsorb appreciable amounts of the compounds although it is not silanised. The chief purpose of silanising the conical tube is to improve the shape of the interface when the last of the aqueous layer is being removed.

On the recommendation of Carnis et al. [5] for clomipramine and desmethylclomipramine, 0.01 *M* HCl was adopted as the aqueous solvent for all the compounds. However, these solutions have not been found easy to handle. On pouring them into a series of tubes, from one to the next, adsorptive losses have been encountered on polystyrene, on glass and to a lesser extent on silanised glass. It seems possible that ion pair formation in the acid solution may be involved. Of the six drugs used here, clomipramine and desmethylclomipramine are the most strongly adsorbed. Unlike the bases, in which the adsorption on glass and on poor column packings is much stronger for the secondary amine than for the related tertiary amine, the adsorption of the salts

is approximately equal for secondary and tertiary, but depends markedly upon the tricyclic nucleus. Silanising the 15-ml tube used in the extraction is essential to minimise salt adsorption. In preparing and diluting the standard solutions in 0.01 *M* HCl, the glassware is silanised, small vessels are avoided and pipettes are thoroughly pre-rinsed with solution to saturate their surfaces.

Extraction of the bases from the heptane layer into 2 ml of 0.1 *M* HCl appears to be complete. In three experiments, compounds were sought in the discarded heptane layer:

(1) The heptane was evaporated to dryness and the residue taken up in solvent and injected into the chromatograph. Pentane was also tried instead of heptane in this experiment.

(2) The heptane was re-extracted with a further 2 ml of 0.1 *M* HCl which was processed and chromatographed as usual.

(3) Using very high drug concentrations, 10 μl of the discarded heptane layer was injected directly.

None of the six drugs was found in any experiment, although in each case it was shown that a few percent of the original amount of any of them would have been detected.

It was also found that neither the yield nor the peak area ratios were altered when an ordinary HCl layer was re-extracted successively with two extra 10-ml volumes of heptane, before proceeding with further treatment. Thus any ion pair formation in the acid solution does not result in significant losses of the compounds in the heptane phase.

The dilute aqueous solutions to be added to plasma were at one time divided out into 10-ml polystyrene tubes and frozen. They were then thawed daily as required. However, in about 20% of these tubes large erratic losses were encountered, particularly of dextromethorphan. Solutions have since been stored successfully in flasks at 4°.

CONCLUSIONS

The precision and accuracy of this method have been tested over a long period, and with nearly 700 estimations, as shown in Tables II—IV. The sensitivity could probably be improved by using the Perkin-Elmer nitrogen detector [20], albeit at considerable cost. However, as the internal standards must remain chemically different from the drugs estimated, it is not clear whether in routine practice the processes of extraction and chromatography could be made reproducible enough to take advantage of any large increase in sensitivity.

The present method has been used successfully for pharmacokinetic studies both in patients undergoing treatment with amitriptyline or nortriptyline and in normal subjects after single doses of amitriptyline. Because the results are not affected by the volume of plasma, large samples can be used if available, to increase precision at low concentrations.

We believe that the method could readily be adopted by other laboratories without further developmental work.

ACKNOWLEDGEMENTS

We wish to thank Dr. Eric Bailey for excellent advice on gas chromatography, Dr. Susan Chesters for valuable advice and help with statistics, and Dr. R. P. Hullin for support and encouragement. We thank the Medical Research Council for a project grant, for recurrent expenses and support of M.A.R.

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